Rabbit Erythrocyte Hemolysis by Lipophilic, Aryl Molecules* (33737)

KENNETH S. ROGERS
(Introduced by Edwin S. Higgins)
Department of Biochemistry, Medical College of Virginia, Richmond, Virginia 23219

Hansch and Fujita (1) have shown that biological activity for various aromatic compounds may be correlated with such physicochemical parameters as partition coefficients and Hammet *sigma* values (a chemical reactivity index). In certain cases (2) either index alone may correlate with some specific biological activity. In the present report, an equation is presented that correlates lysis of rabbit red blood cells with the lipophilicity of indole, skatole, and certain other aromatic molecules. These observations seem to agree with existing information on hemolysis by alkyl reagents (3).

Materials and Methods. Erythrocytes were collected at 0° by centrifugation, 600*g*, from fresh, heparinized ear vein blood from New Zealand male rabbits that weighed about 2 kg. The rabbits were maintained on Purina chow and water *ad libitum*. The red blood cells were washed 3 times in isotonic saline prior to collection for use in the lysis experiments. The number of red blood cells used in the lytic assay was estimated by microscopic counting and also by use of a Coulter counter. The absorbance for an erythrocyte–saline suspension in a cuvette of 1 cm light path was 1.95 at 500 mμ. This value was equivalent to 33,000 red blood cells/mm^3.

Aryl compounds, dissolved in propylene glycol, were added to 1.4×10^8 erythrocytes in saline to give 5.0 ml of a 10% propylene glycol, 0.9% NaCl mixture. After incubation for 60 min at 38°, the suspension was centrifuged at 0° for 20 min at 1000*g*. The extent of hemolysis was determined by absorbance at 540 mμ and also by the procedure of Ponder (4). The two methods were in agreement for detecting 100% hemolysis.

Partition coefficients of aromatic compounds were determined for the *n*-octanol:50 m*M* sodium phosphate (pH 7.4) system. The *n*-octanol was washed with 1 *M* Na_2CO_3 and collected by distillation at 194° and redistillation of the product. Following a procedure somewhat modified from that of Hansch *et al.* (2), 2 ml of *n*-octanol, saturated with buffer, and containing 4 mg of the dissolved aryl compound, were added to 10 ml of phosphate buffer saturated with *n*-octanol. The two phases were mixed at 25° by rotation for 12 hr and then were separated by centrifugation at 25° for 2 hr at 600*g*. The concentration of aryl compound in each phase was determined by ultraviolet spectroscopy and the partition coefficient was calculated (ratio of octanol solubility to aqueous phase solubility). Altering the relative proportions of octanol to buffer did not affect the partitioning of the aryl compounds.

Results and Discussion. In 1937, Forbes and Neal (5) reported that intracapsular injection of indole into the knees of rabbits produced a chronic arthritis. This work has been confirmed and extended to the action of other tryptophan metabolites and aromatic compounds.[1] Since the type of tissue damage suggests a membrane interaction with indole, skatole, and other aryl molecules, red blood cells of rabbits were used to investigate the stability of cellular membranes in the presence of these aromatic reagents. Extent of erythrocyte lysis was measured by loss of cellular shape (4) and release of hemoglobin. Susceptibility of these rabbit red blood cells to lysis by sodium lauryl sulfate was identical to results reported by Ponder (6), indicating that our method of obtaining these cells apparently did not significantly affect their membrane stability.

The relationship between the minimum concentration of aryl molecules to produce 100% hemolysis under our experimental con-

* Supported by a grant from the John A. Hartford Foundation.

[1] Irene Nakoneczna, J. C. Forbes, and K. S. Rogers, unpublished data, manuscript in preparation for publication.

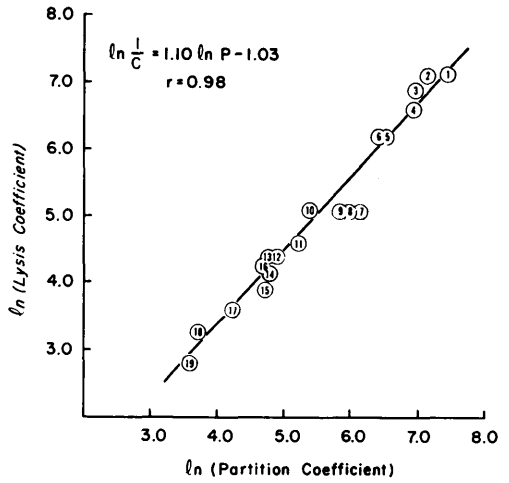

FIG. 1. Lysis of rabbit red blood cells by lipophilic, aryl molecules. The lysis coefficient ($1/C$) is the reciprocal of the minimum molar concentration of aromatic compound required for 100% hemolysis; partition coefficients (P) are for the *n*-octanol:aqueous phosphate (pH 7.4) system. Additional experimental detail is given in the text. 1, diphenylamine; 2, thianaphthene; 3, naphthalene; 4, 5-bromoindole; 5, 1, 2-dimethylindole; 6, indene; 7, 5-methylindole; 8, 3-methylindole; 9, 2-methylindole; 10, 5-cyanoindole; 11, indole; 12, toluene; 13, 5-methoxyindole; 14, quinoline; 15, anisole; 16, benzothiazole; 17, indazole; 18, benzoxazole; 19, benzene. The regression coefficient (r) is for the fit of experimental points to the equation given in the figure.

ditions and the molecules' partition coefficients as indices of their lipophilicity is expressed in the form of a least squares linear regression equation that is given in Fig. 1. The regression value for the correlation of experimental points to the line is 0.98. Thus, the minimum concentration of aryl molecules required to produce total lysis of 1.4×10^8 erythrocytes is inversely proportional to the lipophilicity of the molecules. The equation in Fig. 1 is similar to those given by Hansch and Fujita (1) for correlating partition coefficients with biological activities. In this case, it is thought that the action of these hemolytic, aryl molecules takes place on or in the cell membrane after a single partitioning from a hydrophilic to a lipophilic phase has occurred. Thus, the equation in Fig. 1 presents the expected results. Furthermore, the relative order and magnitude by which the aryl molecules produce hemolysis apparently does not reflect a charge transfer interaction with the membrane since the order for charge complex formation (7) does not parallel our results.

The importance of lipophilicity in the lytic action of these aromatic molecules agrees with the report that diphenylamine (a very good hemolytic reagent, Fig. 1) is absorbed by the membrane lipid fractions in various bacteria (8). Moreover, if the nonpolar character of the red blood cell membrane is increased, due to binding of the aryl molecules, then facilitation of hemolysis might occur through a decrease in the hydration of the erythrocyte membrane since Arrhenius activation values for lysis depend more on the hydration structure of the membrane than on any other single factor (9).

In conclusion, indole, skatole (3-methylindole), and other aromatic molecules can interact with cellular membranes and, by virtue of their lipophilic properties, produce hemolysis of rabbit erythrocytes.

Summary. Indole, skatole, and 17 additional aryl molecules can interact with the cellular membrane to produce hemolysis of the rabbit erythrocyte. The minimum concentrations of aryl molecules required to produce total lysis of 1.4×10^8 red blood cells are correlated with the lipophilicity of the molecules as measured by their partitioning across the interface barrier of *n*-octanol:aqueous phosphate buffer, 50 m*M*, pH 7.4, phases. Hemolysis requires lesser amounts of a molecule that is lipophilic than one that is hydrophilic.

1. Hansch, C. and Fujita, J., J. Am. Chem. Soc. **86**, 161 (1964).

2. Hansch, C., Muir, R. M., Fujita, T., Maloney, P. P., Geiger, F., and Streich, M., J. Am. Chem. Soc. **85**, 2817 (1963).

3. Bodansky, M., J. Biol. Chem. **79**, 241 (1928).

4. Ponder, E., "Hemolysis and Related Phenomena," pp. 30–225. Grune and Stratton, New York, (1948).

5. Forbes, J. C. and Neal, R. C., J. Lab. Clin. Med. **22**, 921 (1937).

6. Ponder, E., J. Gen. Physiol. **30**, 15 (1946).

7. Kanner, L. C. and Kozloff, L. M., Biochemistry **3**, 215 (1964).

8. Salton, M. R. J. and Smith, M. D., Biochim. Biophys. Acta **135**, 196 (1967).
9. Coldman, M. F. and Good, W., Biochim. Biophys. Acta **150**, 194 (1968).

Received Nov. 15, 1961. P.S.E.B.M., 1969, Vol. 130.

Restoration of Immunologic Competence of Neonatally Thymectomized Mice by Isogeneic and Xenogeneic Thymic Grafts (33738)

GEORGE A. HALLENBECK, THEODORE P. KUBISTA[1] AND ROY G. SHORTER
Mayo Clinic and Mayo Foundation: Section of Organ and Tissue Transplantation, Rochester, Minnesota 55901

The importance of the thymus in the development of immunologic competence has been established in experiments on animals of many species (1). Neonatally thymectomized mice of many strains fail to develop normal immune responses, and the capacity of these animals to form humoral antibody, to reject allogeneic skin grafts, and to repopulate their lymphoid systems can be restored by grafts of isogeneic or allogeneic thymus transplanted as free grafts or in "Millipore" diffusion chambers (2–6). Attempts to restore the immunologic capacity of neonatally thymectomized mice by use of zenogeneic grafts of rat thymus have given varied results. Yunis *et al.* (7) reported that, although subcutaneous grafts of thymus from newborn Holtzman rats survived in neonatally thymectomized C3H mice, the grafts did not alter the wasting disease, the depletion of lymphoid tissue, or the inability of splenic cells to mount a graft-against-host reaction. Law (8), however, found that subcutaneous grafts of thymic tissue from Osborn-Mendel rats or from a Syrian hamster restored immunologic competence in neonatally thymectomized F_1 hybrid mice (C57BL/KA × C3HF/Lw).

Having shown previously that the Walker 256 carcinosarcoma of rats grows only briefly, if at all, in intact C57BL/6 mice but usually flourishes in neonatally thymectomized mice (9, 10), we performed experiments to determine whether grafts of isogeneic thymus or grafts or rat thymus could restore the capacity of such thymectomized mice to reject the tumor.

Methods and Procedure. Mice of the C57BL/6 strain were maintained in an air-conditioned environment at 70 to 72°F, with free access to a commercial chow[2] and to water. Thymectomy was performed in each mouse less than 24 hr after birth by the method of Sjodin and his associates (11) using hypothermia for anesthesia. Intact mice and sham-thymectomized mice served as controls. When 3 weeks of age each mouse was injected subcutaneously in the lumbodorsal region with approximately 5×10^6 Walker tumor cells suspended in Tyrode's solution and prepared as described previously (9). Aliquots of each batch of tumor cells were injected into two Sprague-Dawley rats to be certain that the cells were viable.

Groups of neonatally thymectomized mice received grafts of sliced thymic or other tissues intraperitoneally when 2 weeks of age, 1 week before being inoculated with tumor cells. Grafted tissue included thymus from mice or rats, spleen, skeletal muscle, liver, duodenum, lung, kidney, submaxillary glands, and thyroid glands from mice 2 weeks of age and spleen from mice 2 weeks old that had undergone thymectomy when less than 24 hr of age. Thymus and spleen were implanted both free and in Millipore chambers prepared as described by Amos (12) and using filters of 0.1-μpore size. In one series of tests, thymic tissue was killed before transplantation by freezing five times

[1] Mayo Graduate School of Medicine, University of Minnesota, Rochester: Resident in Surgery.

[2] Rock Island Rat and Mouse Food, Monmouth, Ill.